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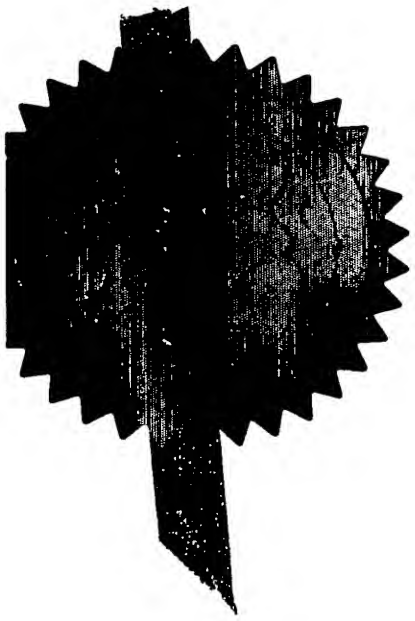

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   Proteome Sciences plc
   Coveham House
   Downside Bridge Road
   Cobham
   Surrey KT11 3EP

   07671670001
   Patents ADP number *(if you know it)*

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4. Title of the invention — Screening Methods

5. Name of your agent *(if you have one)*

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Screening Methods

Field of the Invention

The present invention relates to methods for screening for substances capable of modulating the phosphorylation of tau protein, and in particular paired helical filament (PHF) tau, and the use of such modulators in the treatment of tauopathies. The assays and screening methods are based on the identification of new phosphorylation sites in PHF tau and new kinases and combinations of kinases as therapeutic targets.

Background of the Invention

Alzheimer's disease (AD) is a neurodegenerative disease characterised by the presence of senile plaques and neurofibrillary tangles in the brain. The degree of dementia at death correlates better with neurofibrillary tangle numbers than with senile plaques counts. The presence of neurofibrillary tangles in neurons results in the death of those neurons, implying that prevention of tangle formation is an important therapeutic goal. The principal protein that forms the neurofibrillary tangle is the microtubule-associated protein, tau, which assembles into filaments that have the appearance of twisting about each other in pairs and are referred to as paired helical filaments (PHF). PHF are present in different locations in degenerating neurons in the Alzheimer brain and when many aggregate in the neuronal cell body, they produce the neurofibrillary tangle (Lee et al, 2001).

Senile plaques have an extracellular central deposit of amyloid β-peptide (Aβ), which is surrounded by dystrophic neurites to form the senile or neuritic plaque. *In vitro*

and *in vivo* Aβ has been shown to be neurotoxic. Aβ is derived by proteolytic processing of the larger amyloid precursor protein (APP). Much attention has been focused on Aβ production as a therapeutic target because its production is believed to be an early event in AD pathogenesis. This is because mutations in the APP gene, which give rise to autosomal dominant AD, result in either increased overall production of Aβ or in a relative increase in the slightly longer $A\beta_{42}$ over $A\beta_{40}$, the former being more amyloidogenic; $A\beta_{42}$ has two additional hydrophobic amino acids at the C-terminus of 40-residue $A\beta_{40}$ thereby endowing the peptide with an increased tendency to aggregate and form amyloid fibres. Mutations in two other genes that also cause autosomal dominant AD, presenilin-1 and presenilin-2 (PS1 & PS2) also result in an increase in the ratio of $A\beta_{42}$ to $A\beta_{40}$. The belief that Aβ deposition in the brain precedes the appearance of neurofibrillary tangles has been the basis of the amyloid cascade hypothesis but it has been uncertain whether tangles are important in pathogenesis or are only an unimportant epiphenomenon. This has been changed by the discovery of mutations in the gene for tau in some other related neurodegenerative diseases.

The mechanism by which Aβ kills neurons in the brain has still to be established. Many studies of Aβ toxicity have been conducted in tissue culture using rat brain neuronal cultures. We have shown that exposure of both foetal rat and human brain neuronal cultures to aggregated Aβ induces within 2 to 10 minutes increases in the phosphotyrosine content of several proteins but also including tau (Williamson et al 2002). We have also shown that this treatment results in activation of the

tyrosine kinase fyn, a member of the src family of tyrosine kinases. This tyrosine phosphorylation of tau was prevented by inhibitors of the src family of tyrosine kinases.

It has previously been reported that increased levels of fyn are associated with neurons containing abnormally phosphorylated tau in AD brain (Shirazi et al, 1993) and we have demonstrated using antibodies that recognise phosphotyrosine that PHF-tau from AD brain contains phosphotyrosine (Williamson et al 2002). We have shown *in vitro* that fyn and Lck, both src family kinases, phosphorylate recombinant human tau and phosphotyrosines 18, 310 and 394 were positively identified in one or more of their respective tryptic peptides, from sequence information of fragmented peptides. In addition, phosphotyrosine at position 197 was inferred from peptide masses in the survey scan (Scales et al, 2002).

Intraneuronal deposits of tau in the form of typical neurofibrillary tangles of AD or other morphologically distinct tau aggregates in a number of other neurodegenerative diseases, is the basis for grouping these conditions as tauopathies. Thus, in addition to AD, the main examples of the tauopathies are frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), progressive supranuclear palsy (PSP), Pick's disease, corticobasal degeneration, and multisystem atrophy (MSA). The intracellular tau deposits (usually neuronal but can also be glial) are all filamentous and mostly in a hyperphosphorylated state compared to the level of phosphorylation of tau from control human brain. In the case of AD, this

hyperphosphorylated tau is often referred to as PHF-tau because it is derived from the PHF.

Other than for AD, deposits of Aβ in the brain are either absent or minimal in these other tauopathies. There are some tauopathy pedigrees with autosomal dominant disease in which the causative gene has been identified as the tau gene and although cases with the same mutation may present with apparently different diseases, they invariably have tau deposits in the brain and are mostly of the FTDP-17 variety. Thus, the finding of mutations in the tau gene which result in disease and deposition of tau aggregates in neurons is compelling evidence for the primary pathogenic importance of tau deposition in all of these conditions, including AD, whatever the primary cause of disease. Therefore, the amyloid cascade hypothesis is borne out by the discovery of tau mutations and confirms that indeed neurofibrillary tangle formation is almost certainly subservient to Aβ deposition in AD, but that in the other tauopathies lacking Aβ deposits, then some other primary event must trigger the tau pathology. Tau abnormalities and deposition are therefore important therapeutic targets for all tauopathies, including AD.

Tau is a phosphoprotein, the function of phosphorylation remaining to be unequivocally established. However, increased phosphorylation of tau on multiple serine and threonine residues reduces the ability of tau to promote microtubule assembly and to stabilise assembled microtubules, effects that have been demonstrated both *in vitro* and in cells. Many studies have shown that PHF-tau from AD brain is more heavily phosphorylated on serine and threonine than tau from control brain. This has been

demonstrated partly by protein sequencing and partly by demonstrating that certain monoclonal antibodies only label either PHF-tau or non-phosphorylated tau and not PHF-tau; the epitopes for many of these antibodies have been mapped to particular phosphorylated residues present in PHF-tau and absent from control brain tau. The pathological tau from most other cases of other tauopathies seems to be similarly hyperphosphorylated to PHF-tau.

These findings strongly imply that similar abnormalities in regulating phosphorylation of tau are shared by all the tauopathies including AD. Since phosphorylation of proteins is effected by protein kinases and dephosphorylation by protein phosphatases, identifying the protein kinases and phosphatases for tau is important because they are potentially therapeutic targets for these diseases.

It remains a considerable problem in the art in identifying the enzymes responsible for causing phosphorylation of paired helical filament tau and the sites phosphorylated by those enzymes.

Summary of the Invention

Broadly, the present invention relates to the modulation of the phosphorylation of tau protein through its interaction with kinases and phosphatases. In particular, it is based on the identification of new sites in tau protein that are susceptible to phosphorylation by kinases and to the identification of kinases and combinations of kinases that are capable of phosphorylating new and known phosphorylation sites in tau protein. Importantly, many of the newly identified

sites are present in paired helical filament (PHF) tau, and not in control tau or fetal tau.

The present invention is based on the analysis by mass spectrometry PHF-tau and tau from control adult and foetal rat brain, and identifies 12 new sites in PHF-tau, bringing the total to 37 phosphorylation sites (1 site is tyrosine 394 and the other 36 are either serine or threonine residues) and this with >90% sequence coverage. Of these 12 sites, 11 have not been found in tau from normal human brain.

A number of protein kinases have been demonstrated to phosphorylate tau *in vitro*, including glycogen synthase kinase-3α (GSK-3α), glycogen synthase kinase-3β (GSK-3β), MAP kinases (ERKs 1 & 2), cdk5, cdc2 kinase, JNK, several members of the SAP kinases (1γ, 2a, 2b, 3, 4), p38MAP kinase, calmodulin-dependent kinase, protein kinase A (PKA), protein kinase C (PKC), casein kinase 1 (CK1), casein kinase 2 (CK2), MARK, PKN, PKB, TTK, DYRK and phosphorylase kinase. Of these kinases, GSK-3 has been demonstrated to phosphorylate the greatest number of identified sites in PHF-tau, this being 25 sites, including 2 sites that are generated by GSK-3 only when tau is already phosphorylated and PKA phosphorylates 16 sites in PHF-tau. We have now shown also by *in vitro* phosphorylation that CK1 is also a candidate kinase for 6 of the 12 newly identified sites, GSK-3 phosphorylates 4 of these and PKA phosphorylates 2 of the new PHF-tau sites. This brings the total number of sites in PHF-tau that can be phosphorylated by CK1 to 17 sites.

The MAP kinases (ERKs 1 & 2), cdk5, cdc2 kinase, JNK, several members of the SAP kinases (1γ, 2a, 2b, 3, 4),

and p38MAP kinase are similar in specificity to GSK-3, being essentially proline-directed protein kinases and they all phosphorylate most of the sites phosphorylated by GSK-3. Thus, after these proline-directed protein kinases, CK1 is now the most conspicuous kinase as a candidate for contributing to generating the phosphorylation state of PHF-tau, with only 13 CK1 sites definitely shared by GSK-3. Therefore, of the 36 ser/thr sites in PHF-tau, 31 could potentially be phosphorylated by a combination of GSK-3, CK1, and PKA, and the additional 5 sites remain as orphan sites with no kinase known to phosphorylate these residues. It is possible that GSK-3, CK1 or PKA could phosphorylate some or all of these orphan sites or indeed that one or more of the other potential tau kinases listed above could be responsible and the phosphorylated sites have not been detected. However, the data disclosed herein imply that CK1 should be considered as a strong candidate for generating hyperphosphorylated tau in AD and the other tauopathies and hence is a potential therapeutic target.

Of the known phosphorylation sites in PHF-tau, several are considered to be particularly important. Monoclonal antibody, AT100, of all such antibodies is the most specific for PHF-tau since it does not recognise normal brain tau nor foetal tau; as such it is considered to be diagnostic for pathological hyperphosphorylated tau in the tauopathies. The AT100 epitope requires phosphorylation of both T212 and S214. It is known that T212 and S214 can be phosphorylated by GSK-3 and it has been reported that phosphorylation T212 by GSK-3 primes tau for phosphorylation at S214 by PKA (Singh et al, 1995a,b). We have found that CK1 is also able to

phosphorylate S214, thereby further implicating CK1 in pathological phosphorylation.

One other site in tau, S262, has been shown to be important in regulating the binding of tau to microtubules such that phosphorylation causes dissociation of tau. A novel kinase, MARK, that phosphorylates tau at this site was isolated from brain and proposed as the responsible kinase. We have found that CK1 is also able to phosphorylate S262 and S356, the latter being an homologous residue that may behave like S262 in contributing to regulating binding of tau to microtubules and we have found that both S262 and S356 are phosphorylated in PHF-tau.

Thus, the above two classes of phosphorylation of tau that are considered to be important could be regulated by CK1. Furthermore, it has been reported that CK1, particularly the CK1δ isoform, is elevated in brain extracts from AD cases compared to controls, which adds to the potential importance of CK1 in pathogenesis (Ghoshal et al, 1999).

With respect to tyrosine phosphorylation, PHF-tau is phosphorylated on tyrosine 394 and fyn is the strongest candidate although other src family kinases may also phosphorylate tau in brain.

Accordingly, in one aspect, the present invention proposes that CK1 is a novel therapeutic target for treating AD and other related tauopathies.

In a further aspect, the present invention proposes that fyn and related src family kinases are novel therapeutic

targets for treating AD and other related tauopathies, in particular for tyrosine phosphorylation sites disclosed herein.

In a further aspect, the present invention proposes new phosphorylation sites in tau protein for use in screening for inhibitors of phosphorylation or promoters of dephosphorylation, optionally used in combination with the kinases identified herein as being capable of phosphorylating the sites.

As a consequence of these findings, the new sites and kinases can be used as the basis of assays and assays methods for screening for modulators of the phosphorylation of the sites in tau protein for use or development as therapeutics for the treatment of tauopathies. Preferred modulators are capable of inhibiting the phosphorylation of PHF tau and/or promoting the dephosphorylation of phosphorylated forms of PHF tau.

Eleven of the new phosphorylation sites in tau protein are shown in Table 2 in red type in the left hand column. They are the serine and threonine residues at positions S68, T69, T71, (T111/S113), S191, S258, S289, (T414/S416), T427, S433 and S435. A further tyrosine site at position 394 (Y394) has also been identified (e.g. phosphorylated by tyrosine kinases and dephosphorylated by tyrosine phosphatases). Of the 12 sites, 10 are only found in PHF tau, see Table 2 comparing the PHF tau and control tau columns.

Accordingly, in a further aspect, the present invention provides the use of a tau protein comprising one or more

of these phosphorylation sites as defined herein for screening for candidate substances which are capable of inhibiting phosphorylation at the site(s) by a kinase or promoting dephosphorylation of a phosphorylated site by a phosphatase.

In the present invention, the tau protein comprising the phosphorylation sites may be substantially full length and/or wild type tau or PHF tau protein, or may be a fragment, active portion or sequence variant thereof. In other embodiments, the present invention may employ a corresponding nucleic acid molecule encoding the tau protein. Where a tau protein which is a fragment, active portion or sequence variant is employed, the phosphorylation site(s) may be present with surrounding amino acids from the tau protein sequence. Preferably, the present invention employs PHF tau protein. In the present invention the numbering of tau and PHF tau is according to the sequence disclosed in Goedert et al (1989) EMBO J. 1989 Feb;8(2):393-9. Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNAs in human brain. Goedert M, Spillantini MG, Potier MC, Ulrich J, Crowther RA.

Alternatively or additionally, any of the above defined tau proteins may possess phosphorylation at one or more of the phosphorylation sites. This enables the effects of cooperative phosphorylation of the protein to be studied, that is, where the phosphorylation of one site is dependent in changes to the tau protein caused by one or more preceding or simultaneous phosphorylation steps. Thus, in some embodiments of the present invention, the

tau protein may include one or more of the known tau phosphorylation sites, for example those set out in Table 2, left hand column in black type, in addition to one or more of the newly found sites, and optionally have phosphorylation at one or more of those additional sites.

In a further aspect, the present invention provides a method of screening for substances which are capable of inhibiting phosphorylation at one or more of the site(s) of a tau protein by a kinase, wherein the tau protein comprises one or more phosphorylation sites disclosed herein, the method comprising:

(a) contacting at least one candidate substance, the tau protein as defined herein and a kinase which is capable of phosphorylating the tau protein under conditions in which the kinase is capable of phosphorylating the site(s) of the tau protein in the absence of the candidate substance;

(b) determining whether, and optionally the extent to which, the candidate substance inhibits the phosphorylation of the tau protein at one or more sites of the tau protein; and,

(c) selecting the candidate substance which inhibits phosphorylation of the tau protein at one or more of the sites.

In a further aspect, the present invention provides a method of screening for substances which are capable of promoting dephosphorylation at one or more of the site(s) of a tau protein by a phosphatase, wherein the tau protein comprises one or more sites as defined herein, the method comprising:

(a) contacting at least one candidate substance, the tau protein as defined herein and a phosphatase which

is capable of dephosphorylating the tau protein under conditions in which the phosphatase is capable of dephosphorylating the site(s) of the tau protein in the absence of the candidate substance;

(b) determining whether, and optionally the extent to which, the candidate substance promotes the dephosphorylation of the tau protein at one or more sites of the tau protein; and,

(c) selecting the candidate substance which promotes dephosphorylation of the tau protein at one or more of the sites.

In some embodiments, the method may comprise, having identified a candidate substance according to one of the methods disclosed herein, the further step(s) of optimising the candidate substance to improve one or more of its properties and/or formulating it as a pharmaceutical.

In the methods and uses disclosed herein, preferably the kinase is selected from casein kinase 1 (CK1), casein kinase 2 (CK2), protein kinase A (PKA), glycogen synthase kinase 3α (GSK-3α), and glycogen synthase kinase 3β (GSK-3β). More preferably, the kinase is CK1 or a combination (either simultaneously or sequentially applied) of CK1, PKA and GSK-3β.

In the present invention, preferably the step of detecting the presence and extent of phosphorylation and dephosphorylation in the tau protein can be carried out using mass spectroscopy as described in detail below. Alternatively, or additionally, site specific recognition agents which are capable of distinguishing between a site which is phosphorylated and one which is not may be used.

Examples of such agents known in the art are site specific antibodies such as monoclonal antibody AT100.

In a further aspect, the present invention provides a substance obtainable from one of the methods disclosed herein which is capable of inhibiting the phosphorylation or promoting the dephosphorylation of a tau protein at one or more of the above defined sites.

A further aspect of the present invention is based on the finding that casein kinase 1 is capable of phosphorylating a tau protein at previously unknown positions. Some of the positions are known or suspected in the art of being phosphorylation sites, while others are among the phosphorylation sites identified herein for the first time. The sites of PHF-tau protein that are phosphorylated by CK1 include (S46/T50), S113, S131, T149, T169, S184, S208, (S210/T212), S214, S237, S238, S241, S258, S262, S285, S289, S305, S341, S352, S356, T361, T373, T386, (S412/S413/T414), S416, S433 and S435. Of these sites, S113, S258, S289, S433 and S435 are disclosed as phosphorylation sites of PHF-tau protein for the first time herein. The sequence of casein kinase 1 is provided in J Biol Chem. 1993 Mar 25;268(9):6394-401. Molecular cloning, expression, and characterization of a 49-kilodalton casein kinase I isoform from rat testis. Graves PR, Haas DW, Hagedorn CH, DePaoli-Roach AA, Roach PJ.

Accordingly, the present invention provides the use of a casein kinase 1 as defined herein (including fragments, active portions or sequence variants), or a corresponding nucleic acid molecule, for screening for candidate compounds which are capable of (a) inhibiting the

activity of casein kinase 1 in phosphorylating a tau protein such as paired helical filament tau or (b) binding to casein kinase 1 to inhibit its interaction with a tau protein such as paired helical filament tau.

In a further aspect, the present invention provides a method of screening for substances which are capable of inhibiting the phosphorylation of a tau protein by casein kinase 1 (CK1), wherein the tau protein comprises one or more phosphorylation sites disclosed herein, the method comprising:

(a) contacting at least one candidate substance, the tau protein as defined herein and casein kinase 1 under conditions in which the casein kinase 1 is capable of phosphorylating the site(s) of the tau protein in the absence of the candidate substance;

(b) determining whether, and optionally the extent to which, the candidate substance inhibits the phosphorylation of the tau protein at one or more sites of the tau protein by casein kinase 1; and,

(c) selecting the candidate substance which inhibits phosphorylation of the tau protein at one or more of the sites.

In a further aspect, the present application also discloses that a combination of kinases is required to phosphorylate the majority of the phosphorylation sites disclosed herein or in the prior art. In the experiments disclosed herein, a combination of casein kinase 1 (CK1), protein kinase A (PKA) and glycogen synthase kinase 3β (GSK-3β) was found to be capable, either alone or in cooperation, of phosphorylating the majority of the phosphorylation sites of tau protein and in particular PHF tau protein. This combination of kinases can be used

simultaneously or sequentially to screen for modulators of tau phosphorylation, in contrast to prior art proposals that have focussed on screening using a single kinase.

Accordingly, the present invention provides the use of a casein kinase 1 (CK1), protein kinase A (PKA) and glycogen synthase kinase 3β (GSK-3β) (including fragments, active portions or sequence variants), or a corresponding nucleic acid molecule, for screening for candidate compounds which are capable of (a) inhibiting the activity of casein kinase 1 in phosphorylating a tau protein such as paired helical filament tau or (b) binding to casein kinase 1 to inhibit its interaction with a tau protein such as paired helical filament tau.

In a further aspect, the present invention provides a method of screening for substances which are capable of inhibiting the phosphorylation of a tau protein by casein kinase 1 (CK1), protein kinase A (PKA) and glycogen synthase kinase 3β (GSK-3β), wherein the tau protein comprises one or more phosphorylation sites disclosed herein, the method comprising:

(a) contacting at least one candidate substance, the tau protein as defined herein and casein kinase 1 (CK1), protein kinase A (PKA) and glycogen synthase kinase 3β (GSK-3β) under conditions in which the kinases are capable of phosphorylating the site(s) of the tau protein in the absence of the candidate substance;

(b) determining whether, and optionally the extent to which, the candidate substance inhibits the phosphorylation of the tau protein at one or more sites of the tau protein by the kinases; and,

(c) selecting the candidate substance which inhibits phosphorylation of the tau protein at one or more of the sites.

In this aspect of the invention, one or more of these kinases may be substituted by a kinase having the same or a similar activity and/or substrate specificity.

Embodiments of the present invention will now be discussed in more detail by way of example and not limitation with reference to the accompanying tables.

Tables

Table 1 summarises the new sites found in the work leading to the present invention and the kinases capable of acting at those sites. Tables 2 and 3 present this data in more detail.

Detailed Description

Tau proteins

The assays and assay methods disclosed herein can employ wild-type or full length tau proteins, kinases or phosphatases or fragments, active portions or derivatives thereof. In the case of tau proteins, the materials used in the assays may be unphosphorylated or partially phosphorylated as discussed above.

In the present invention, derivatives of the tau proteins, kinases (especially CK1 kinase) or phosphatases have an amino acid sequence which differs by one or more amino acid residues from the wild-type amino acid sequence, by one or more of addition, insertion, deletion and substitution of one or more amino acids. Thus, variants, derivatives, alleles, mutants and homologues,

e.g. from other organisms, are included. Thus, a derivative of tau protein or CK1 kinase may include 1, 2, 3, 4, 5, greater than 5, or greater than 10 amino acid alterations such as substitutions with respect to the wild-type sequence.

Preferably, a fragment or derivative of a protein used in the assays disclosed herein preferably shares sequence identity with the corresponding portion of the relevant wild-type sequence of the protein, and preferably has at least about 60%, or 70%, or 75%, or 80%, or 85%, 90% or 95% sequence identity. As is well-understood, identity at the amino acid level is generally in terms of amino acid identity which may be defined and determined by the TBLASTN program, of Altschul *et al.* (1990) *J. Mol. Biol.* **215**: 403-10, which is in standard use in the art. Identity may be over the full-length of the relevant peptide or over a contiguous sequence of about 5, 10, 15, 20, 25, 30, 35, 50, 75, 100 or more amino acids, compared with the relevant wild-type amino acid sequence. Alternatively, nucleic acid encoding a fragment or derivative may hybridise to the corresponding wild type nucleic acid under stringent conditions, for example as disclosed in textbooks such as Ausubel, Short Protocols in Molecular Biology, 1992 or Sambrook et al, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989, using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 0.5-1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and

0.1% SDS; (3) 30 minutes-1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is (Sambrook et al., 1989):

T_m = 81.5°C + 16.6Log [Na+] + 0.41(% G+C) - 0.63 (% formamide) - 600/#bp in duplex

As an illustration of the above formula, using [Na+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C. Such a sequence would be considered substantially homologous to the nucleic acid sequence of the present invention.

Methods of Screening for Inhibitors and Enhancers

It is well known that pharmaceutical research leading to the identification of a new drug may involve the screening of very large numbers of candidate substances, both before and even after a lead compound has been found. This is one factor which makes pharmaceutical research very expensive and time-consuming. Means for assisting in the screening process can have considerable commercial importance and utility.

As detailed above, methods of screening for a substance which are inhibitors of phosphorylation of tau protein or promoters of dephosphorylation of tau protein can be

carried out by contacting one or more test substances with the tau protein and kinase or phosphatase (as defined herein) in a suitable reaction medium, and determining the presence or extent of phosphorylation of dephosphorylation in the presence and absence of the candidate substance. A difference in activity in the presence and absence of the candidate substance is indicative of a modulating effect.

Preliminary assays *in vitro* may be followed by, or run in parallel with, *in vivo* assays.

Of course, the person skilled in the art will design any appropriate control experiments with which to compare results obtained in test assays.

Performance of an assay method according to the present invention may be followed by isolation and/or manufacture and/or use of a compound, substance or molecule which tests positive for ability to modulate interaction between one of the phosphorylation sites of tau protein (as defined herein) and a kinase (such as CK1 or a combination of CK1, PKA and GSK-3β) or a phosphatase.

The precise format of an assay of the invention may be varied by those of skill in the art using routine skill and knowledge. For example, interaction between substances may be studied *in vitro* by labelling one with a detectable label and bringing it into contact with the other which has been immobilised on a solid support. Suitable detectable labels, especially for peptidyl substances include ^{35}S-methionine which may be incorporated into recombinantly produced peptides and polypeptides. Recombinantly produced peptides and polypeptides may also be expressed as a fusion protein

containing an epitope which can be labelled with an antibody.

The protein which is immobilized on a solid support may be immobilized using an antibody against that protein bound to a solid support or via other technologies which are known *per se*. A preferred *in vitro* interaction may utilise a fusion protein including glutathione-S-transferase (GST). This may be immobilized on glutathione agarose beads. In an *in vitro* assay format of the type described above a test compound can be assayed by determining its ability to diminish the amount of labelled peptide or polypeptide which binds to the immobilized GST-fusion polypeptide. This may be determined by fractionating the glutathione-agarose beads by SDS-polyacrylamide gel electrophoresis. Alternatively, the beads may be rinsed to remove unbound protein and the amount of protein which has bound can be determined by counting the amount of label present in, for example, a suitable scintillation counter.

The amount of a candidate substance which may be added to an assay of the invention will normally be determined by trial and error depending upon the type of compound used. Typically, from about 0.001 nM to 1mM or more concentrations of putative inhibitor compound may be used, for example from 0.01 nM to 100μM, e.g. 0.1 to 50 μM, such as about 10 μM. Greater concentrations may be used when a peptide is the test substance. Even a molecule which has a weak effect may be a useful lead compound for further investigation and development.

Combinatorial library technology provides an efficient way of testing a potentially vast number of different

substances for ability to modulate activity of a polypeptide. Such libraries and their use are known in the art. Compounds which may be used may be natural or synthetic chemical compounds used in drug screening programmes. Extracts of plants which contain several characterised or uncharacterised components may also be used.

Antibodies directed to the site of interaction in either protein form a further class of putative inhibitor compounds. Candidate inhibitor antibodies may be characterised and their binding regions determined to provide single chain antibodies and fragments thereof which are responsible for disrupting the interaction. Antibodies may also be employed as site specific recognition agents for determining whether phosphorylation of a site in tau protein has occurred during as assay.

Antibodies may be obtained using techniques which are standard in the art. Methods of producing antibodies include immunising a mammal (e.g. mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage *et al.*, 1992, Nature 357: 80-82). Isolation of antibodies and/or antibody-producing cells from an animal may be accompanied by a step of sacrificing the animal.

As an alternative or supplement to immunising a mammal with a peptide, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO 92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with any of the proteins (or fragments), or may be one constructed using sequences obtained from an organism which has been exposed to the antigen of interest.

Antibodies according to the present invention may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any binding substance having a binding domain with the required specificity. Thus the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimicks that of an antibody enabling it to bind an antigen or epitope.

Example antibody fragments, capable of binding an antigen or other binding partner are the Fab fragment consisting of the VL, VH, Cl and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain; isolated CDR regions and F(ab')2 fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

A hybridoma producing a monoclonal antibody according to the present invention may be subject to genetic mutation or other changes. It will further be understood by those skilled in the art that a monoclonal antibody can be subjected to the techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP 0 184 187 A, GB 2 188 638 A or EP 0 239 400 A. Cloning and expression of chimeric antibodies are described in EP 0 120 694 A and EP 0 125 023 A.

Hybridomas capable of producing antibody with desired binding characteristics are within the scope of the present invention, as are host cells, eukaryotic or prokaryotic, containing nucleic acid encoding antibodies (including antibody fragments) and capable of their expression. The invention also provides methods of production of the antibodies including growing a cell capable of producing the antibody under conditions in which the antibody is produced, and preferably secreted.

The reactivities of antibodies on a sample may be determined by any appropriate means. Tagging with individual reporter molecules is one possibility. The reporter molecules may directly or indirectly generate detectable, and preferably measurable, signals. The linkage of reporter molecules may be directly or indirectly, covalently, e.g. via a peptide bond or non-covalently. Linkage via a peptide bond may be as a

result of recombinant expression of a gene fusion encoding antibody and reporter molecule. The mode of determining binding is not a feature of the present invention and those skilled in the art are able to choose a suitable mode according to their preference and general knowledge.

Other candidate inhibitor compounds may be based on modelling the 3-dimensional structure of a polypeptide or peptide fragment and using rational drug design to provide potential inhibitor compounds with particular molecular shape, size and charge characteristics.

Development of Mimetic Substances

Once candidate substance have been found in the assays and screens according to the present invention, they may be used to design mimetic compounds for development as drugs. The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are unsuitable active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing is generally used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target

property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, eg spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this in the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or

modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

Pharmaceutical Compositions

Following identification of a substance which modulates or affects phosphorylation or dephosphorylation of tau protein, the substance may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

Thus, the present invention extends in various aspects not only to a substance identified using the screening assays and assay methods disclosed herein, but also a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method comprising administration of such a composition to a patient, e.g. to treat tauopathies, use of such a substance in manufacture of a composition for administration for the treatment of tauopathies, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

The substances identified as kinase inhibitors or phosphatase promoters in the assays and assay methods of the present invention, or compounds or substances arising from further development or optimisation, may be formulated in pharmaceutical compositions. These compositions may be employed for the treatment of tauopathies, that is conditions which are characterised by

neurofibrillary tangles or aggregates of tau protein. Tauopathies are a recognised class of conditions known to those skilled in the art and include Alzheimer's disease (AD), frontotemproal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), progressive supranuclear palsy (PSP), Pick's disease, corticobasal degeneration and multisystem atrophy (MSA). The intracellular tau deposits are usually neuronal or glial and are filamentous and generally in a hyperphosphorylated state as compared to the level of phosphorylation in tau from control human brain. In the case of AD, this hyperphosphorylated tau is often referred to a paired helical filament tau (PHF) tau because it is derived from the PHF.

These compositions may comprise, in addition to one of the above substances, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Whether it is a polypeptide, antibody, peptide, nucleic acid molecule, small molecule or other pharmaceutically useful compound according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 20th Edition, 2000, pub. Lippincott, Williams & Wilkins. A composition may be administered alone or in combination with other

treatments, either simultaneously or sequentially, dependent upon the condition to be treated.

Materials and Methods

Mass Spectrometry

Data Acquisition

Following SDS-PAGE the gel bands relating to PHF-tau were excised, reduced, alkylated and digested with trypsin. Peptides were extracted from the gel pieces by a series of acetonitrile and aqueous washes. The extract was pooled with the initial supernatant and lyophilised. Each sample was then resuspended in 6 ml of 50mM ammonium bicarbonate and analysed by LC/MS/MS. Chromatographic separations were performed using an Ultimate LC system (Dionex, UK). Peptides were resolved by reverse phase chromatography on a 75 mm C18 PepMap column. A gradient of acetonitrile in 0.05% formic acid was delivered to elute the peptides at a flow rate of 200 nl/min. Peptides were ionised by electrospray ionisation using a Z-spray source fitted to a Q-Tofmicro (Micromass, UK). The instrument was set to run in automated switching mode, selecting precursor ions based on their intensity, for sequencing by collision-induced fragmentation.

Data Analysis

The mass spectral data was processed into peak lists and searched against the full-length sequence of Tau-6 (441 amino acids; mw 45847) using Mascot software (Matrix Science, UK). Phosphorylated peptides were identified by selecting phosphate as a variable modification within the searching parameters. Serine, threonine and tyrosine phosphorylation were all considered. The exact location of the modification within each peptide was determined by the pattern of fragment ions produced (see below for

Preparation and purification of recombinant human tau

A plasmid expressing the largest tau isoform (2N4R) was used to prepare and purify recombinant human tau as described previously (Mulot et al, 1994). Briefly, a bacterial cell lysate expressing 2N4R tau was heated and centrifuged to remove heat-labile proteins. The supernatant was fractionated with ammonium sulphate and precipitated material was solubilised and dialysed into buffer prior to cation-exchange chromatography. Proteins were eluted with NaCl and fractions containing tau were pooled and dialysed against ammonium bicarbonate before lyophilisation.

In vitro phosphorylation of recombinant tau by serine-/threonine protein kinases

Recombinant human tau (40µg/ml) was incubated with 67U/ml casein kinase 1 (CK1), 67U/ml casein kinase 2 (CK2), 167U/ml cyclic AMP-dependent protein kinase (PKA), 67U/ml glycogen synthase-3β (GSK-3β) or all four kinases in combination, each at the stated concentration, in the presence of 3mM ATP for 6h at 30°C. Each kinase was obtained in a purified recombinant form from New England Biolabs.

In-gel proteolytic digestion of tau

PHF-tau or *in vitro* phosphorylated tau proteins were separated on 10 % (wt/vol) polyacrylamide gels and stained with colloidal Coomassie Blue G. Protein bands corresponding to tau were excised, carbamidomethylated, and digested with proteolytic enzymes (trypsin or Asp-N). Peptides were extracted from gel pieces by a series of acetonitrile and aqueous washes, dried and resuspended in 50mM ammonium bicarbonate.

Amyloid beta treatment of neurons

Rat and human cortical neurons were treated with Aβ peptide (25-35) or reverse Aβ peptide (35-25) for 1-10 min. Proteins containing phosphotyrosine were immunoprecipitated and separated by SDS-PAGE. Western blots of heat-stable extracts of neuronal cultures and immunoprecipitates were probed with antibodies to tau.

Results

New sites found in PHF-tau

Current literature reports 25 known phosphorylation sites (all are serine or threonine) identified by direct means in PHF-tau (Hanger et al, 1998). There are a further 2-3 sites that have been identified by antibody reactivity only. We have found an additional 12 phosphorylation sites in PHF-tau, one of which is a tyrosine residue (tyr394), bringing the total number of sites to 37. Four of the new sites are more amino terminal in tau than any previously reported sites and three sites are more carboxy terminal than found previously. Of the 12 new sites, 4 are present in alternatively-spliced regions of tau and therefore are present only in specific tau isoforms, all previously identified PHF-tau phosphorylation sites are present in all tau isoforms. Only one of the 12 new sites in PHF-tau (either thr414 or ser416) is detected in tau from normal brain (ser416).

New sites on recombinant tau for each of the 4 serine/threonine kinases investigated

See also Table 1

CK1 found 29 new sites making a total of 31 sites in all.

17 CK1 sites are present in PHF-tau, including 15 of the new CK1 sites. CK1 is a candidate kinase for 6 of the 12 new PHF-tau sites.

CK2 found 5 new sites making a total of 8 in all. 5 CK2 sites are present in PHF-tau, including 3 of the new CK2 sites. CK2 is a candidate kinase for 1 of the 12 new PHF-tau sites

GSK-3 found 13 new sites making a total of 39 in all. 25 GSK-3 sites are present in PHF-tau, including 5 of the new GSK-3β sites. GSK-3 is a candidate kinase for 4 of the 12 new PHF-tau sites

PKA found 5 new sites making a total of 24 in all. 16 PKA sites are present in PHF-tau, including 4 of the new PKA sites. PKA is a candidate kinase for 2 of the 12 new PHF-tau sites

Comparing PHF-tau phosphorylation sites with the recombinant tau and kinase data, when all of the phosphorylation sites for CK1, GSK-3β, and PKA are combined, 27-30 of the 37 PHF-tau sites are phosphorylated (3 sites are defined only as one of two adjacent residues). Of the residual 7 sites, one is a tyrosine residue that requires tyrosine kinase activity, 5 have no known kinase and the remaining site is phosphorylated by GSK-3 if tau is pre-phosphorylated. These results suggest that a combination of kinases is required to bring about a phosphorylation state that resembles PHF-tau.

Seven of the 12 new phosphorylation sites in PHF-tau could be generated by CK1, GSK-3, or PKA, four have no

known kinase and the fifth site required a tyrosine kinase for phosphorylation.

Combining the four kinases together in a single reaction, we generated one site (thr111) that was not detected with any of the four kinases alone, this residue is not phosphorylated by any other known kinase *in vitro*. Phosphorylation at this residue is also present in PHF-tau. These results show that combinations of kinases can result in phosphorylation at new sites, possibly due to conformational changes induced by the primary phosphorylation step that increase the likelihood of the secondary phosphorylation, possibly by a second enzyme.

Amyloid beta treatment of neurons

We found that treatment of neurons with Aβ peptide increased tyrosine phosphorylation of neuronal proteins including tau. The increase in phosphotyrosine induced by Aβ was approximately four times the basal level in tau.

Future experiments

Identify phosphorylation sites of other individual and combinations of protein kinases to emulate PHF-tau phosphorylation *in vitro*. Kinases that have been implicated in tauopathies include GSK-3α, ERKs 1 & 2, cdk5, cdc2 kinase, JNK, several members of the SAP kinase family (1γ, 2a, 2b, 3, 4), p38MAP kinase, calmodulin-dependent kinase, PKC, MARK, PKN, PKB, TTK, DYRK and phosphorylase kinase.

Determine if phosphorylation of tau with these kinases and other tyrosine kinases induces tau aggregation *in vitro* and in cells. This will allow us to identify the

phosphorylation sites that are critical for tau aggregation.

Investigate the effects of specific protein kinase inhibitors, alone and in combination, on tau aggregation in an *in vitro* or cellular context.

Generate transgenic mice (inducibly) expressing CK1 and determine if this model shows cerebral tau deposition. Cross this mouse with other mice expressing candidate kinases (eg a GSK-3 mouse already exists) and examine the rate of tangle formation.

We have recently found (unpublished) that tyr394 is phosphorylated in AD and in foetal tau and have reported that this same residue is phosphorylated by both Fyn and Lck *in vitro*. Fyn has been shown previously to phosphorylate tau and Fyn is increased in a sub-set of neurons in AD. It is also known that Aβ treatment of neurons induces tau phosphorylation and that Fyn knock-out mice are resistant to Aβ.

We will treat neurons from wild-type, Fyn knock-out and Src knock-out mice with Aβ and identify the phosphorylation sites on tau in each case.

It is possible that other tyrosine kinases are involved in tau phosphorylation and aggregation and these include those associated with growth factor and neurotrophic factor receptors. Other tyrosine kinase families may also be involved, including Syk kinase, which has been show to phosphorylate another protein (α-synuclein) implicated in neurodegenerative disease in a manner that increases its propensity to aggregate *in vitro*. In each

case, we will investigate the effects of phosphorylation on tau aggregation and the effects of kinase inhibition on tau aggregation.

5

Table 1: New sites identified in tau as phosphorylated by individual serine/threonine kinases

Kinase	New sites identified in recombinant tau	New sites present in PHF-tau	Kinase sites present in 12 new PHF-tau sites
CK1	(46/50), 113, 131, 149, 169, 184, 208, (210/212), 214, 237, 238, 241, 258, 262, 263, 285, 289, 305, 341, 352, 356, 361, 373, 386, (412/413/414/416, 2 sites), 416, 433, 435	113, 184, 208, (210/212), 214, 237, 238, 258, 262, 289, 356, (412/413/414/416, 2 sites), 416, 433, 435	(111/113), 258, 289, 416, 433, 435
CK2	(52/56), 199, 386, 400, (412/413/414/416, 1 site)	199, 400, (412/413/414/416, 1 site)	(412/413/414/416, 1 site)
GSK-3	149, 220, 237, 241, 245, 258, 285, 289, 305, 352, 373, (409/412/413/414/416, 3 sites of which 2 or 3 are new)	237, 258, 289, (409/412/413/414/416, 3 sites of which 2 or 3 are new)	258, 289, (409/412/413/414/416, 3 sites of which 2 or 3 are new)
PKA	210, (217/220), 258, 352, (412/413)	210, (217/220), 258, (412/413)	258, 416

The 12 new sites in PHF-tau are:
68, 69, 71, (111/113), 191, 258, 289, Y394, (414/416), 427, 433, 435

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The references mentioned herein are all expressly incorporated by reference.

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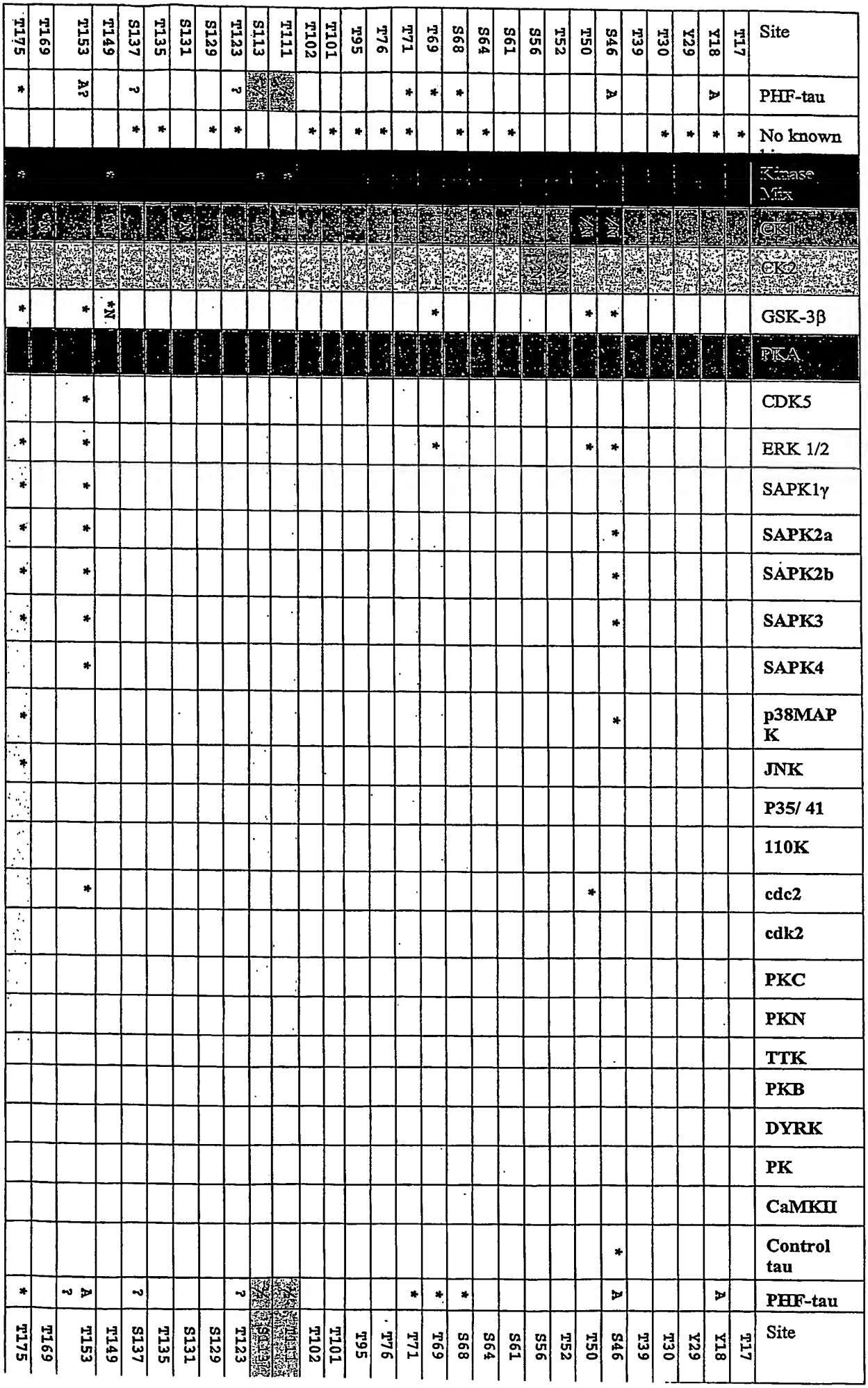
Site
PHF-tau
No known
Kinase Mix
CK1
CK2
GSK-3β
PKA
CDK5
ERK 1/2
SAPK1γ
SAPK2a
SAPK2b
SAPK3
SAPK4
p38MAPK
JNK
P35/ 41
110K
cdc2
cdk2
PKC
PKN
TTK
PKB
DYRK
PK
CaMKII
Control tau
PHF-tau
Site
T17
Y18
Y29
T30
T39
S46
T50
T52
S56
S61
S64
S68
T69
T71
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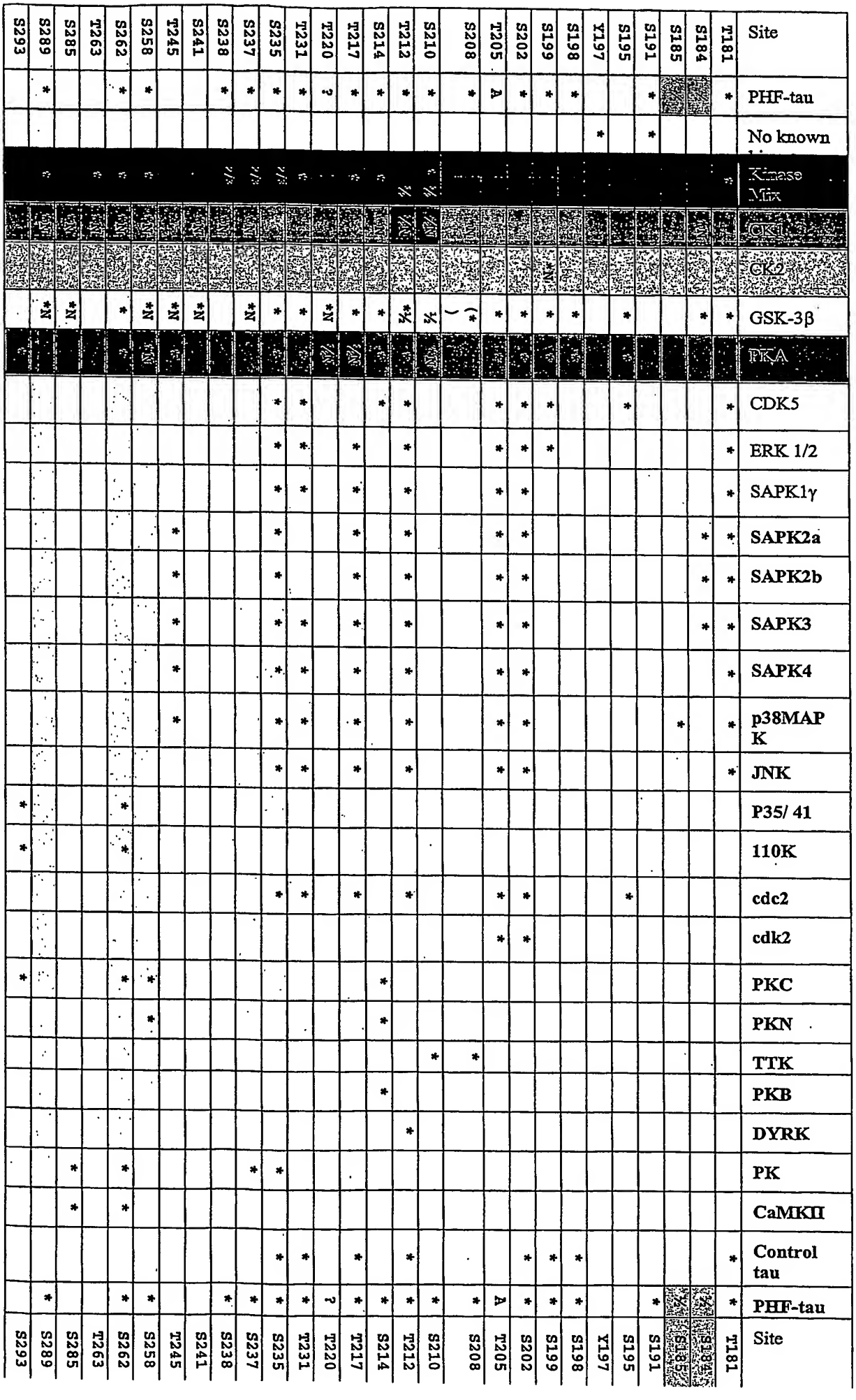
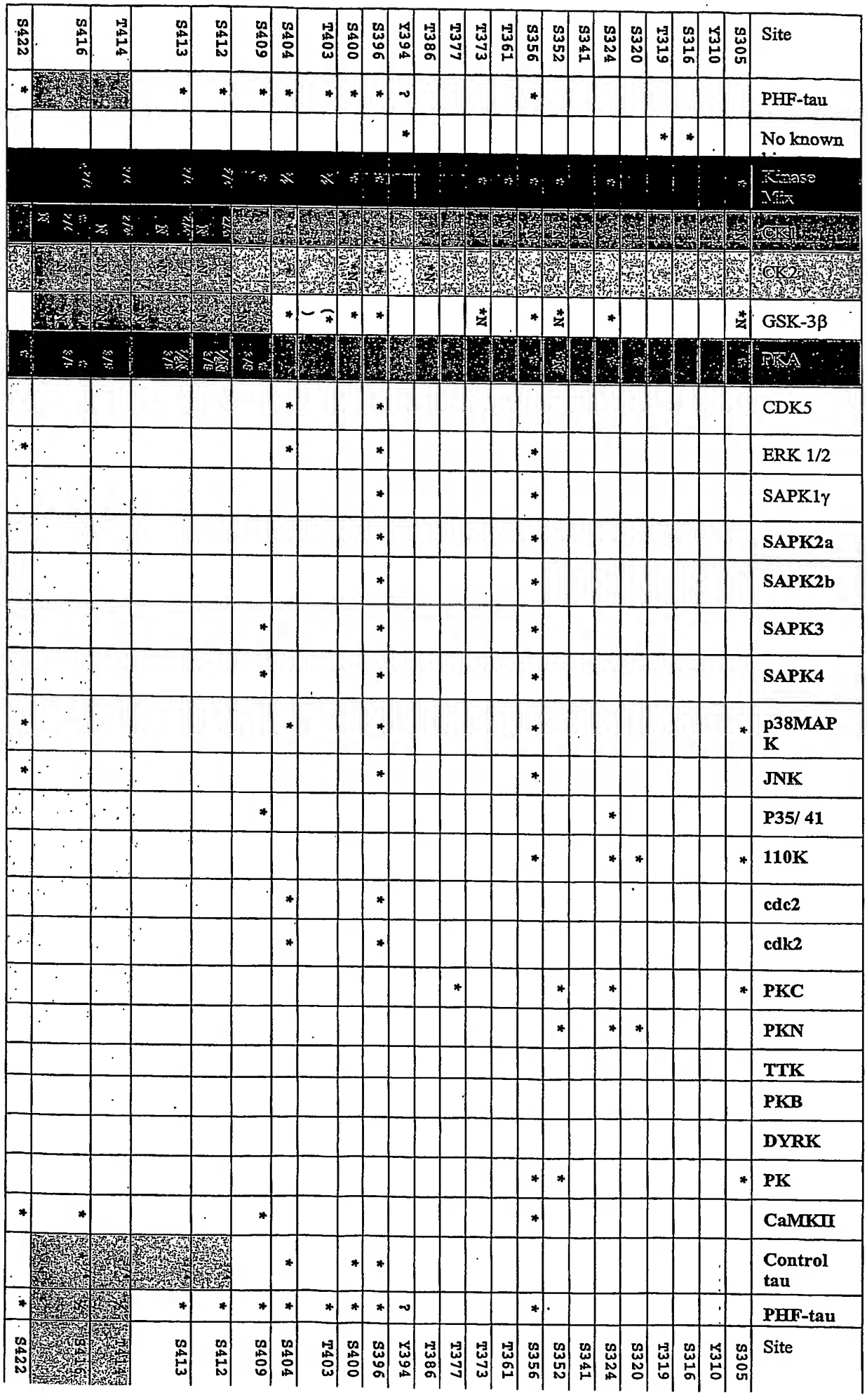
T102
S113
T123
S129
S131
T135
S137
T149
T153
T169
T175


Site
PHF-tau
No known
Kinase Mix
CK1
CK2
GSK-3β
PKA
CDK5
ERK 1/2
SAPK1γ
SAPK2a
SAPK2b
SAPK3
SAPK4
p38MAPK
JNK
P35/ 41
110K
cdc2
cdk2
PKC
PKN
TTK
PKB
DYRK
PK
CaMKII
Control tau
PHF-tau
Site
T181
S184
S185
S191
S195
Y197
S198
S199
S202
T205
S208
S210
T212
S214
T217
T220
T231
S235
S237
S238
S241
T245
S258
S262
T263
S285
S289
S293


Site
PHF-tau
No known
Kinase Mix
CK1
CK2
GSK-3β
PKA
CDK5
ERK 1/2
SAPK1γ
SAPK2a
SAPK2b
SAPK3
SAPK4
p38MAPK
JNK
P35/ 41
110K
cdc2
cdk2
PKC
PKN
TTK
PKB
DYRK
PK
CaMKII
Control tau
PHF-tau
Site
S305
Y310
S316
T319
S320
S324
S341
S352
S356
T361
T373
T377
T386
Y394
S396
S400
T403
S404
S409
S412
S413
T414
S416
S422

Site	PHF-tau	No known	Kinase Mix	CK1	CK2	GSK-3β	PKA	CDK5	ERK 1/2	SAPK1γ	SAPK2a	SAPK2b	SAPK3	SAPK4	p38MAPK	JNK	P35/41	110K	cdc2	cdk2	PKC	PKN	TTK	PKB	DYRK	PK	CaMKII	Control tau	PHF-tau	Site
T427	*	*																											*	T427
S433	*		½	A																									*	S433
S435	*		½	N																									*	S435

* = identified phosphorylation site
A = identified by antibody labelling; ½ etc = one of two adjacent sites phosphorylated in PHF-tau; ? = suspected sites in PHF-tau
New PHF-tau sites indicated in red. Ubiquitination at: K254, K257, K311, K317
"Control tau" includes combined data from human and rat tau

Kinase phosphorylation sites

Shaded sites indicate multiple (numbered) phosphorylations at less well-defined sites (see below), N indicates a new site.

Some of the kinase phosphorylation sites were identified using antibodies (eg GSK-3β, S208)
Sites identified by MS or antibodies are all compiled together in this table
(*) = GSK-3β requires priming at Thr212 before Ser208 is phosphorylated and priming at Ser404 before Thr403 is phosphorylated
TTK = tau tubulin kinase; DYRK = dual-specificity tyrosine phosphorylated and regulated kinase

Ambiguous Sites (not all are included here)

CK1
46/50 =1/2 possible sites
210/212 =1/2
412/413/414/416 =2/4
416/422 =1/2

CK2
52/56 =1/2
412/413/414/416 =1/4

PKA
409/412/413/414/416 =3/5

GSK-3
403/404 =1/2
409/412/413/414/416 =2/5

Kinase Mix (=CK1, CK2, GSK-3β, PKA)
210/212 =1/2
210/212/214/217 =3/4

235/237/238	=2/3
400/403/404	=2/3
403/404	=1/2
412/413/414/416	=2/4
433/435	=1/2

New Sites

CK1:	28	(of which 3 are ambiguous)
CK2:	5	(of which 2 are ambiguous)
GSK-3β:	12	(of which 1 is ambiguous)
PKA:	5	(of which 2 are ambiguous)

Table 2

Site	PHF -tau	No known kinase	CK1 GSK-3 PKA	[illegible] kinases	CK1	CK2	GSK-3β	PKA	Control tau	PHF-tau	Site
S68	*	*								*	S68
T69	*		*				*			*	T69
T71	*	*								*	T71
T111											T111
S113			*								S113
T175	*		*				*			*	T175
T181	*		*				*		*	*	T181
S184			*				*				S184
S185											S185
S191	*	*								*	S191
S198	*		*				*		*	*	S198
S199	*		*			*N	*		*	*	S199
S202	*		*				*		*	*	S202
S208	*		*				(*)			*	S208
S210	*		*				½			*	S210
T212	*		*				*½		*	*	T212
S214	*		*				*			*	S214
T217	*		*				*		*	*	T217
T231	*		*				*		*	*	T231
S235	*		*				*		*	*	S235
S237	*		*				*N			*	S237
S238	*		*							*	S238
S258	*		*				*N			*	S258
S262	*		*				*			*	S262
S289	*		*				*N			*	S289
S356	*		*				*			*	S356
Y394	?	*								?	Y394
S396	*		*			*	*		*	*	S396
S400	*		*			*N	*		*	*	S400
T403	*		*				(*)			*	T403
S404	*		*			*	*		*	*	S404
S409	*		*							*	S409
S412	*		(*)		N	N	N	N		*	S412
S413	*		*		N	N	N	N		*	S413
T414			(*)		N	N	N				T414
S416			*		N	N	N				S416
S422	*		*							*	S422
T427	*	*								*	T427
S433	*		*							*	S433
S435	*		*							*	S435

Table 3